Targeted transgenesis reveals discrete attenuator functions of GRK and PKA in airway β_2-adrenergic receptor physiologic signaling

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Phosphorylation by protein kinase A (PKA) and G protein-coupled receptor kinases (GRKs) desensitize β_2-adrenergic receptor (β_2AR) signaling, and these are thought to be mechanisms involved with cell and organ homeostasis and tolerance to agonists. However, there is little direct evidence that these events are relevant to β_2AR physiological function, such as airway smooth muscle (ASM) relaxation leading to bronchodilation. To maintain cell- and receptor-specificity without altering the natural complement of kinases/arrestins, transgenic mice were generated expressing the human WT and mutated β_2ARs lacking PKA and/or GRK phosphorylation sites on ASM at ≈4-fold over background. Functional gains in response to β-agonist from the selective loss of these mechanisms were determined in mouse airways. Relaxation kinetics were altered in all mutant airways compared with β_2WT. At low receptor occupancy, β_2PKA(-) had enhanced agonist-promoted relaxation, while β_2GRK(-) airways were unaffected. In contrast, at saturating agonist concentrations, the greatest relaxation enhancement was with β_2GRK(-), with no evidence for additivity when PKA sites were also removed. For the full range of responses, the β_2PKA(-)/GRK(-) airways had the greatest relaxation efficiency, indicating a graded effect of GRKs as agonist concentration increased. ASM cAMP levels paralleled relaxation phenotypes. No interaction between PKA phosphorylation of β_2AR and GRK-promoted events was identified by β-arrestin-2 recruitment. Thus, these two mechanisms indeed impact a relevant β_2AR physiologic function, acting as attenuators of the acute response, and represent specific interfaces where adjunct therapy or biased ligands may improve β-agonist treatment of obstructive lung disease.

desensitization | adenylyl cyclase | smooth muscle | asthma

Desensitization of biologic signaling during activation is a common observation and represents an important component for maintenance of homeostasis within the complex milieu of signals presented to cells and organ systems. Desensitization can also lead to a loss of therapeutic efficacy, often termed tolerance or tachyphylaxis. Defining the mechanisms of desensitization and linking altered cellular signaling to a relevant physiologic function can lead to insights into normal physiologic compensatory events, maladaptive pathologic events in disease, and pharmacologic agents or strategies that display limited tachyphylaxis. Signaling via many G protein-coupled receptors (GPCRs) undergoes rapid agonist-promoted desensitization (1), including the β_2-adrenergic receptor (β_2AR). The regulation of this receptor by agonist has been of particular interest in airway smooth muscle, where β_2AR modulate bronchomotor tone and β_2AR agonists are used for the treatment of airway bronchoconstriction in asthma and chronic obstructive pulmonary disease. Two mechanisms have been rigorously shown in reconstituted (2–4) and cell-based systems (5–7) to mediate the early events of β_2AR desensitization during exposure to agonist: (i) Phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs), with subsequent binding of β-arrestin and its steric attenuation of receptor-G_s coupling, and (ii) phosphorylation by protein kinase A (PKA), which decreases receptor-G_s coupling and promotes receptor-G_i coupling thereby antagonizing the cAMP stimulatory effect of β_2AR. Although these desensitization processes are often discussed in relation to prolonged exposure to agonist, both phosphorylation events occur rapidly (seconds to minutes) after agonist binding (7, 8), and thus should be an integral part of the characteristic immediate physiologic response to agonist, essentially representing built-in attenuators. While reductionist systems have established these kinetic properties (7, 8), there is little data to assess the impact of these events in the initial end-organ response of β_2AR to agonist. A potential additional complexity in transferring the signal presented to the receptor to the physiologic response is based on the concentration of the agonist (the "input signal"). GRK-mediated events require receptor occupancy, whereas PKA activation from a small complement of receptors (i.e., low doses) can heterologously desensitize a potentially larger pool of receptors. While cell-based studies have characterized these mechanisms (5, 6), it has been difficult to ascertain their impact on relevant physiologic functions of a specific receptor expressed on a specific cell-type. For example, a typical (9–11) approach to create a GRK or β-arrestin knockout mouse (i) results in a loss of that protein in all cell-types, (ii) could potentially effect many of the GPCRs that are signaling during a biological event, and (iii) cannot account for potentially analogous functions of the other GRKs or arrestins that are often co-expressed in the same cells. While dominant-negative transgenes have been expressed in specific cell-types (12), the latter two aforementioned issues remain, as well as the specificity of such inhibitors for their intended target protein.

In airway smooth muscle, β_2AR are targets for agonists that act to relax constricted muscle and thus bronchodilate—the only acute bronchodilatory therapeutic available for routine therapy. The use of β_2AR agonists for treating obstructive lung disease has brought about considerable controversy in regards to efficacy as well as adverse events, which may be related to agonist regulation of receptor signaling. Particularly in asthma, acute severe bronchoconstriction is not readily reversed by β-agonists, with administration of dozens of inhalations often required for such patients, regardless of the extent of prior β-agonist use. Thus, understanding the rapid desensitization responses may provide for adjuvant or alternative therapy for reversal of severe acute bronchoconstriction. Furthermore, persistent use of β-agonists has been reported in some studies to be associated with worsening asthma, increased bronchial hyperresponsiveness to constriction, depressed responsiveness to acute β-agonist, and mortality (13). To define the effects and the hierarchy of the GRK- and PKA-mediated pathways for this single GPCR-

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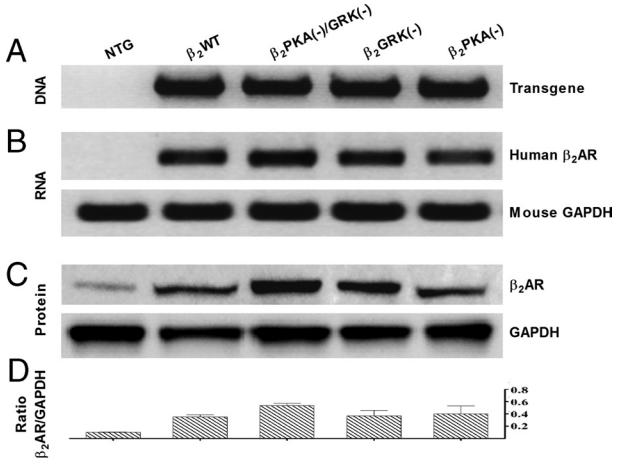

Fig. 1. Generation of transgenic mice expressing β_2AR on airway smooth muscle. (*A*) PCR with primers corresponding to the coding sequence of the human β_2AR and the transgene SV40 terminator show incorporation of the transgene in F2 mouse genomic DNA. (*B*) RT-PCR with human-specific β_2AR primers reveals mRNA expression in cultured airway smooth muscle cells from transgenic but not nontransgenic mice. (*C*) Expression of β_2AR protein in cultured airway smooth muscle cells identified by a species-independent antibody in western blots. (*D*) Results of three western blot experiments (mean ± SE) revealed increased β_2AR expression in airway smooth muscle cells from all four transgenic mice, compared with nontransgenics ($P < 0.01$), with β_2AR overexpression not being different between the various transgenic cells.

subtype, specifically in smooth muscle cells, using a physiologically relevant function (airway relaxation), we constructed mutated β_2AR lacking the GRK and/or PKA phosphorylation sites and generated transgenic mice with expression of these receptors on airway smooth muscle cells.

Results

Generation of Smooth Muscle-Targeted Transgenic Mice. Four groups of transgenic mouse lines (FVB/N strain) were generated expressing the human wild-type (β_2WT) and three mutated β_2ARs on smooth muscle cells using the α-actin smooth muscle promoter and a 3′UTR SV40 polyadenylation terminator sequence (see *Materials and Methods*). The mutated receptors consisted of substitutions of the PKA sites at Ser-261,262 and Ser-345,346 with Ala, and this mouse line is termed β_2PKA(-). The β_2GRK(-) line consists of mice with transgenic expression of the human β_2AR with the 11 Ser and Thr of the cytoplasmic tail, representing all of the potential sites for phosphorylation by GRKs, substituted by Ala. A β_2AR lacking both sets of phosphorylation sites was also transgenically expressed and is termed β_2PKA(-)/GRK(-). Specific transgene incorporation was verified in genomic DNA by using primers that spanned the C-terminal sequence of the β_2AR and the SV40 termination sequence. mRNA expression in primary airway smooth muscle cell lines used RT-PCR primers that are specific for human, but not mouse, β_2AR. As shown in Fig. 1*A*, the transgene was incorporated into the genomes of founder offspring, and mRNA expression in airway smooth muscle was of comparable levels between the transgenic lines, with no signal detected in cells derived from nontransgenic mice (Fig. 1*B*). The background mouse β_2AR expression on airway smooth muscle cells is ≈10 fmol/mg, and our goal was to express the transgenic receptors at levels of 3- to 5-fold over this background. We chose a low level of overexpression because of prior observation (14) that high levels result in up-regulation of phospholipase C and G_q-coupled inositol phosphate signaling. The low levels of protein expression that we sought are below the limits of quantitation by radioligand binding. However, Western blots using concentrated cell protein and a specific β_2AR antibody that does not discriminate between the mouse and human receptors revealed distinct signals, with increased β_2AR expression in the transgenic primary airway smooth muscle cells, compared with nontransgenic. Shown in Fig. 1 *C* and *D* is the β_2AR protein expression from cultured airway smooth muscle cells derived from the mouse lines chosen to study. These results, normalized to GAPDH, revealed a ≈4-fold increase in expression in the transgenics, with no differences in the extent of overexpression between β_2WT, β_2PKA(-), β_2GRK(-), and β_2PKA(-)/GRK(-) in the airway smooth muscle cells (Fig. 1*D*).

PKA- and GRK-Mediated Events Differentially Regulate β_2AR Function in the Intact Airway. Tracheal rings were isolated and studied using the ex vivo isometric myograph method as we have described (15, 16). Rings were passively stretched to 5 mN, then contracted with multiple doses of acetylcholine, and the EC_{50} for acetylcholine contraction was determined. After washout, rings were contracted by continuous exposure to the EC_{50} dose of acetylcholine for the remainder of the experiment, and isoproterenol (ISO) was added to the bath at the indicated concentrations. The extent of relaxation was stable within 5 min after addition of ISO, and the force was recorded for a given dose at this 5-min time point. The results are shown in Fig. 2*A*. Each of the dose-response curves for the four transgenic mice differed from each other as determined by ANOVA ($P < 0.001$). We were particularly interested in the two extremes of the agonist concentrations (Fig. 2*A*, boxes), where posthoc tests compared the extent of relaxation at 1 nM ISO (where PKA-mediated desensitization events were hypothesized to be dominant) and at the saturating, and maximal, concentration of ISO used (30 μM), where GRK effects were hypothesized to be most evident. In β_2PKA(-) rings, relaxation was greater at 1 nM ISO compared with β_2WT, consistent with less desensitization of the β_2PKA(-) compared with β_2WT at this concentration. In marked contrast, β_2GRK(-) rings, which retain the PKA phosphorylation sites, had the identical extent of relaxation at low dose ISO as was found for the β_2WT. Furthermore, removal of GRK phosphorylation sites within the context of absent PKA sites [the β_2PKA(-)/GRK(-) mouse] had no apparent effect on β_2AR-mediated airway relaxation over removal of the PKA sites alone with the 1 nM concentration of ISO, indicating little role for a GRK process in physiologic desensitization when few receptors are occupied.

At the maximal concentration, the β_2GRK(-) rings displayed the greatest relaxation, which was statistically different as compared with β_2PKA(-). The β_2PKA(-)/GRK(-) rings show an apparent response that is intermediate between the two aforementioned mouse rings, but was not statistically different from the β_2PKA(-) response. These results are consistent with GRK phosphorylation events being critical for attenuating the maximal β_2AR responsiveness at high concentrations of agonist and that inhibiting this effect can have significant enhancement of the physiologic action of β_2AR agonists. Furthermore, it appears that the effects of removal of the PKA sites in the context of absent GRK sites is not synergistic, or even additive, at these concentrations of agonist. The signal transduction efficiencies (which take into account the sensitivity and maximal responses to the agonist) were calculated using a weighted Tau function as we previously described (17), so as to ascertain the overall effects of the loss of the various desensitization mechanisms. These values, calculated from the composite curves and normalized to the β_2WT for β_2PKA(-), β_2GRK(-), and β_2PKA(-)/GRK(-), were 22.8, 27.4, and 33.6, respectively, consistent with the greatest overall agonist-promoted physiologic responsiveness (i.e., least amount of desensitization) for β_2PKA(-)/GRK(-) mice. These relative values are intuitively consistent with the morphology of the curves generated from the data as shown in Fig. 2*A*, with the β_2PKA(-)/GRK(-) rings having a response at 30 μM that was nearly equivalent to the β_2GRK(-) rings and a

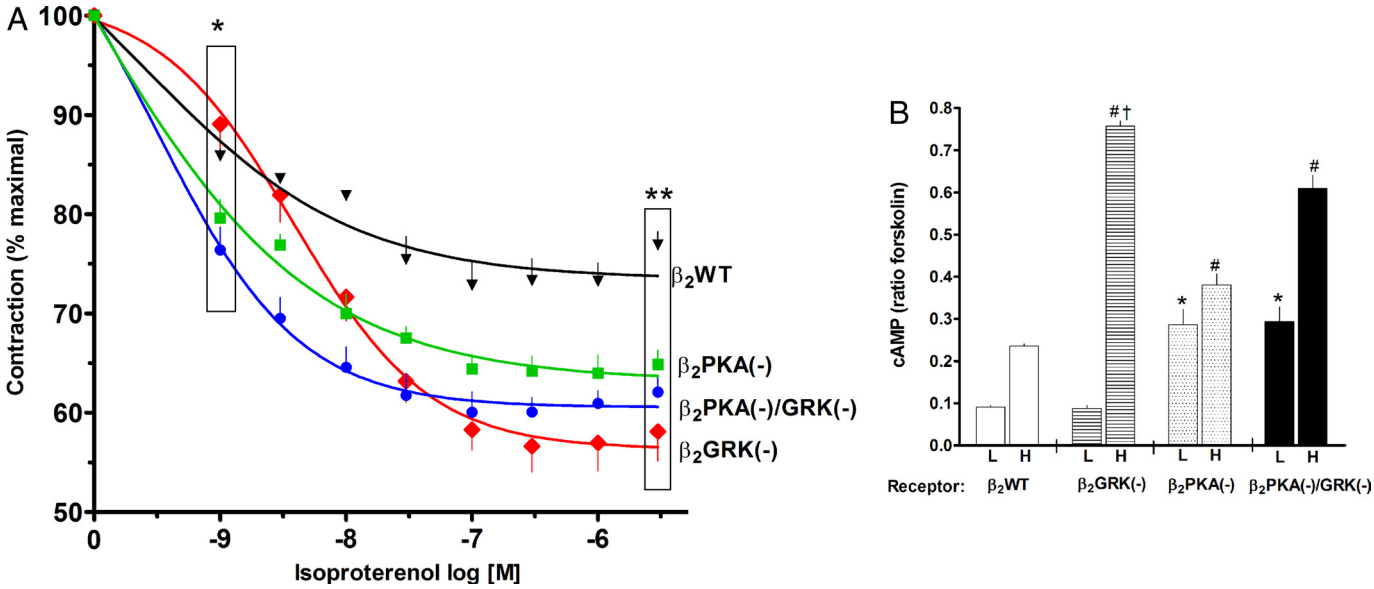

Fig. 2. β_2AR-mediated airway relaxation and airway smooth muscle cAMP phenotypes of receptors lacking GRK and/or PKA phosphorylation sites. (*A*) Airway relaxation in response to ISO from all four mouse lines differed from each other by ANOVA ($P < 0.001$). The boxes indicate responses at the lowest concentration of agonist, where enhanced responsiveness was observed for mice expressing β_2AR lacking PKA sites, or the highest concentrations, where the response was greatest for mice expressing β_2AR lacking GRK sites. Results are mean ± SE from four to six experiments. *, $P < 0.01$ for β_2PKA(-) and β_2PKA(-)/GRK(-) vs. β_2WT. **, $P < 0.01$ for all mutant β_2AR responses vs. β_2WT and $P < 0.05$ for the β_2GRK(-) response vs. β_2PKA(-) or β_2PKA(-)/GRK(-). (*B*) Cells were exposed to low (*L*, 10 nM) or high (*H*, 30 μM) ISO. Results are mean ± SE from four experiments. *, #$P < 0.01$ vs. β_2WT; †, $P < 0.05$ vs. β_2PKA(-)/GRK(-).

left-shift in the dose-response curve as indicated by the greater relaxation at lower concentrations as well.

cAMP Signaling in Airway Smooth Muscle Parallels GRK- and PKA-Mediated Airway Physiology. To ascertain whether intracellular cAMP responses could account for the physiological findings, cAMP was measured in cultured primary airway smooth muscle cells derived from each line in whole-cell assays of attached cells (Fig. 2*B*). At a low concentration of ISO, the β_2GRK(-) cells had the same cAMP levels as β_2WT cells, congruous with the same extent of relaxation observed in the airway physiological studies between these two mice at this concentration (Fig. 2*A*, left-hand box). However, β_2PKA(-) airway smooth muscle cells had ≈3-fold higher agonist promoted cAMP levels at this concentration, again in agreement with the enhanced agonist-promoted relaxation observed for β_2PKA(-) mice. Furthermore, in the β_2PKA(-)/GRK(-) cells, low concentration agonist-stimulated cAMP levels were the same as those from the β_2PKA(-) cells, indicating no additional effect of the GRK mechanism when receptor occupancy is low (Fig. 2*B*). At saturating ISO concentrations, β_2PKA(-) cells revealed higher cAMP production than β_2WT, although this difference was modest. However, the response to β_2GRK(-) cells was markedly higher than β_2WT, and indeed represented the most robust response of all mutant receptors at the saturating concentration (Fig. 2*B*), and is consistent with this mutant receptor having the greatest maximal relaxation (Fig. 2*A*, right-hand box). β_2PKA(-)/GRK(-) cells revealed a cAMP response that was lower than β_2GRK(-) cells, but higher than β_2PKA(-) cells, indicating a potential interaction between the two mechanisms leading to less desensitization than when GRK sites alone are substituted.

Physiologic Phenotypes and Potential Relationships with Histologic Anomalies or Interactions Between Mechanisms. Given that β_2AR activation evokes cAMP production as well as other downstream signals (1), we considered that the airway relaxation phenotypes might be influenced by pathologic remodeling of the airway by one or more of the transgenic receptors. However, as shown in Fig. 3, there was no evidence of smooth muscle hypertrophy or fibrosis, mucous metaplasia, submucosal thickening, or inflammatory cell infiltration in the airways of any of the mutated β_2AR lines compared with β_2WT. Thus, the phenotypes shown in Fig. 2*A* are not readily explained by pathologic effects. We

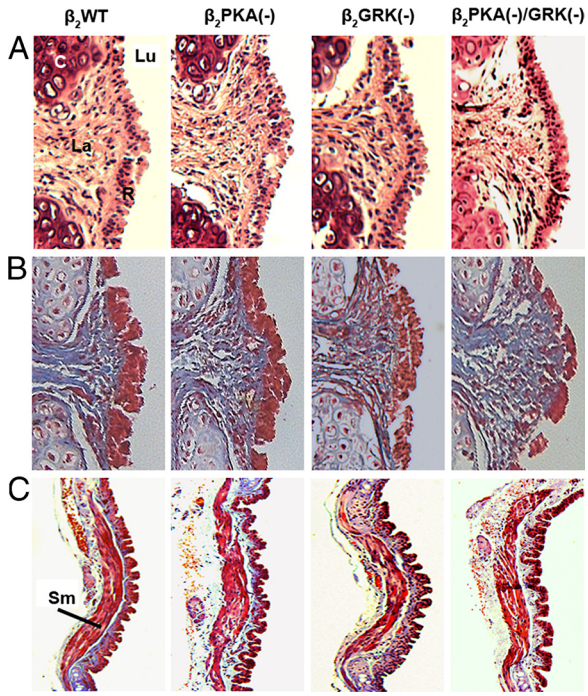

Fig. 3. Airway histology of transgenic mice overexpressing wild-type or mutated β_2AR on airway smooth muscle. Shown are hematoxylin and eosin-stained (*A*) and trichrome-stained (*B* and *C*) longitudinal sections (*A* and *B*) and cross-sections (*C*) of the trachea. Sm, smooth muscle; C, cartilage; E, epithelium; La, Lamina propria; Lu, lumen.

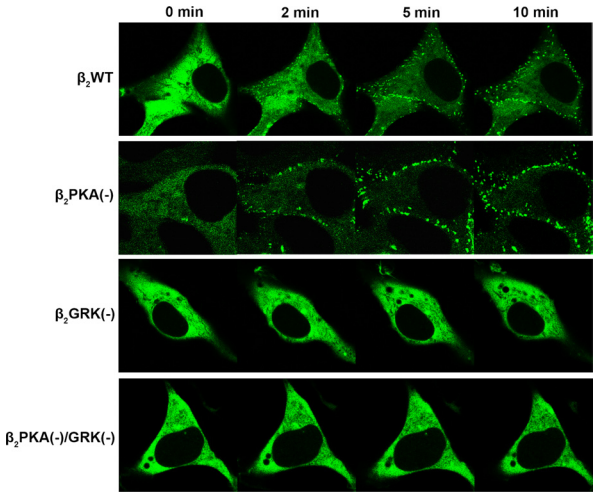

Fig. 4. Effects of substituting PKA and/or GRK sites of β_2AR on agonist-promoted β-arrestin-2 recruitment. HEK-293 cells were transfected to express the indicated β_2AR and β-arrestin-2-GFP, exposed to 10 μM ISO for the times shown, and localization of GFP ascertained by confocal microscopy as described in the *Materials and Methods* section. β_2GRK(-) and β_2PKA(-)/GRK(-) showed no recruitment. β_2PKA(-) displayed recruitment to the cell surface in a manner indistinguishable from β_2WT. Shown is a representative result of four experiments performed.

considered whether interaction between the two desensitization mechanisms could explain why substitution of both the PKA and the GRK sites did not result in a synergistic or additive maximal physiological effect (i.e., greater ISO-promoted relaxation) compared with GRK-substituted receptors. Indeed, an additive effect has been reported for coupling to cAMP in highly overexpressed model cells expressing mutations of the β_2AR that are similar, but not identical, to ours (5). As indicated above, even with unopposed β_2AR signaling in the airway from the absence of both desensitization mechanisms, we found no evidence of smooth muscle pathology or atypical airway architecture, so we cannot evoke an anatomic mechanism for limiting relaxation of the double mutant. We considered that the lack of additivity could be because of an effect of PKA phosphorylation of the receptor on GRK-mediated events. Indeed, the cAMP data from the cultured mouse airway smooth muscle cells are suggestive of such an interaction, since the maximal agonist-promoted cAMP levels were greater for β_2GRK(-) airway smooth muscle cells compared with those from the β_2PKA(-)/GRK(-) mice (Fig. 2*B*). One potential such mechanism is that with wild-type receptor, GRK-mediated functions are enhanced by a PKA event. Given that G$\beta\gamma$ enhances GRK function and PKA-phosphorylated β_2AR couples to G_i releasing $G_i\beta\gamma$, we contemplated whether β-arrestin recruitment could be altered in PKA(-) mutants. We tested this hypothesis using the mutated receptors by transfecting HEK-293 cells with the various β_2AR constructs and β-arrestin-2-GFP and assessed its recruitment to the cell surface during agonist exposure using confocal microscopy. As shown in Fig. 4, we found no recruitment of β-arrestin2-GFP in the β_2GRK(-) or β_2PKA(-)/GRK(-) cells, which was not unexpected, since β_2AR phosphorylation by GRKs is required for recruitment. Furthermore, the recruitment appears to be qualitatively the same between β_2WT and β_2PKA(-) cells, indicating that in the absence of PKA phosphorylation, GRK-mediated recruitment of β-arrestin, the key element of the G_s desensitization process, is unaffected. Finally, we considered whether there were major differences in contraction to acetylcholine evoked by the β_2AR mutations. The acetylcholine pD_2 (- log EC_{50}) values for β_2PKA(-), β_2GRK(-), and β_2PKA(-)/GRK(-) rings were 5.0 $\pm$ 0.16, 4.3 $\pm$ 0.14, and 4.7 $\pm$ 0.10, which were not statistically different from β_2WT (4.7 $\pm$ 0.32, n = 3–6, $P > 0.05$). The maximal contraction was not different between β_2WT, β_2GRK(-) and β_2PKA(-)/GRK(-) rings (27 $\pm$ 0.79, 24 $\pm$ 4.2, and 28 $\pm$ 6.9 mN, respectively). The β_2PKA(-) rings had a lower maximal contraction (17 $\pm$ 3.2 mN, $P < 0.05$) compared with rings expressing the other three receptors. The ISO relaxation phenotype is not, however, affected by the degree of contraction within this range, when relaxation is expressed as a percentage. In initial validation studies, we contracted rings to 25 $\pm$ 1.7 and 18 $\pm$ 1.4 mN (n = 4). ISO-promoted relaxation was very similar under both conditions, amounting to forces of 77 $\pm$ 1.8% and 74 $\pm$ 1.0%, respectively.

Discussion

GPCRs and their regulation serve critical integrative and homeostatic roles in cell and organ function over a wide spectrum of normal physiologic states. However, in disease processes, receptor desensitization can have salutary effects, can be maladaptive and contribute to pathogenesis, or can limit the effectiveness of therapeutic agonists and antagonists. Given the dynamic nature of the signals received by the cell in such perturbed states, we have focused here on the two mechanisms involved in minute-to-minute regulation of the β_2AR, phosphorylation by GRKs and PKA, ascertaining signaling at the cellular level and at the physiologic level of the intact airway. We chose the β_2AR of airway smooth muscle because of its central role in asthma, a disease that displays temporal variability in the extent of airway tone (consistent with the potential need for rapid regulation of GPCRs) and a disease that is treated with β_2AR agonists. As introduced earlier, there is a need for more effective treatments of acute bronchospasm, and agonist therapy itself is associated with adverse effects that may be because of desensitization. To ascertain the contributions of these kinase-mediated events, we generated mice with smooth muscle-targeted expression of the wild-type β_2AR and mutated receptors lacking the GRK and PKA phosphorylation sites. To our knowledge, such an approach that incorporates a single GPCR in the cell-type of interest, without manipulation of the complement of GRKs and arrestins, has not been reported. Using this approach, we could ascertain for this receptor the importance of each mechanism within the context of this relevant physiologic function.

We established several important functional effects of each mechanism and a potential interaction between mechanisms. First, we now show conclusively that at low concentrations of agonist, the PKA-mediated mechanism is the major desensitization pathway at play in airway smooth muscle relaxation. The desensitization by PKA at this low concentration amounted to ≈15%. This extent of desensitization, and the low plasma levels achieved from orally administered β_2AR agonists, may be the basis for the suboptimal response to oral agonists in the treatment of asthma. As a corollary, it would appear that any adjuvant therapy designed to block or minimize GRK-mediated desensitization would not improve efficacy for this treatment scenario. At higher concentrations of agonist, as is observed in the airway with directly inhaled β_2AR agonists, the GRK-mediated effects become apparent, amounting to ≈25% desensitization. Here, a GRK inhibitor would be expected to have a therapeutic impact by improving the extent of bronchodilatation (or antagonizing bronchoconstriction). Or, a biased-ligand (18, 19), in this case one that promotes stabilization of a β_2AR conformation that is favorable for activation of G protein but not for GRK/β-arrestin events, would be predicted to have improved bronchodilatory function. In the treatment of severe bronchospasm, this improved relaxation may eliminate the need for acute administration of cumulatively large doses of inhaled agonist, which causes systemic adverse effects, and, decreased efficacy for subsequent doses at later times in the course of treatment. The loss of both PKA and GRK mechanisms had the expected effect at the low

concentration of agonist from the absence of PKA phosphorylation. As the agonist dose increased, there was a divergence of the β_2PKA(-) and the β_2PKA(-)/GRK(-) curves relative to β_2WT, which is consistent with the progressive influence of GRK-mediated events as more receptors are occupied. At the highest concentration, this difference between the β_2PKA(-) and the β_2PKA(-)/GRK(-) responses is small, which may mean that the two systems serve some degree of functional redundancy (in regards to acute relaxation) that is not additive. Of note, although, the cAMP levels reveal that in fact β_2GRK(-) cells produce more cAMP than the β_2PKA(-)/GRK(-) cells, which may be indicative of PKA phosphorylation of the receptor being required for optimal GRK-mediated desensitization. We have eliminated altered β-arrestin recruitment as a mechanism for this potential interaction. From a physiologic standpoint, this modestly higher cAMP may have little additional functional importance in the acute response, but may be relevant under pathologic conditions where contraction-relaxation dynamics are altered.

The current studies show the relevance of PKA- and GRK-mediated events during acute agonist activation of airway smooth muscle β_2AR, and these results may also be relevant for long-term agonist-promoted regulation of the receptor in airway smooth muscle. The recruitment of β-arrestin to the GRK-phosphorylated receptor is an initial step that leads to receptor internalization into clathrin-coated vesicles, which present the receptor for intracellular degradation during persistent agonist exposure. Also, the PKA-phosphorylated form of the receptor (studied by exposure of cells to permeable cAMP analogs) has been reported to undergo enhanced receptor degradation compared with nonphosphorylated receptor (20). Thus, both mechanisms may be underway during the long-term scenario. However, other major mechanisms of down-regulation of receptor expression from prolonged agonist exposure include decreased gene transcription and decreased mRNA stability, which are dependent, in part, on promoter and 3′UTR sequence (21). The transgenesis method used for the current studies results in random integration of multiple copies of the transgenes into the mouse genome, with expression being driven by a smooth muscle promoter. Thus, studies that involve mechanisms of receptor regulation that include gene transcription and message stability (long-term agonist exposure) are not appropriate for these mice. For such a study, the use of a GRK or β-arrestin knockout mouse could provide information on long-term regulation of the endogenous β_2AR, with the caveats discussed previously concerning other GPCRs being affected, the lack of cell-type specificity, and the remaining native GRKs or β-arrestins serving redundant roles. Such studies have not been reported to date for airway smooth muscle function.

Recently, a β-arrestin-2 knockout mouse has been studied in terms of acute, agonist-mediated, airway smooth muscle relaxation (22). These results revealed that ISO-promoted relaxation of tracheal smooth muscle was enhanced $\approx$10% points in the β-arrestin-2 knockout mouse rings compared with wild-type rings. This effect was not placed into the context of PKA-mediated desensitization, or the combination of both processes, but nevertheless the current study is in agreement with these data in regards to a signal-quenching role for a GRK-associated event in the acute airway smooth muscle relaxation response to β_2AR activation. In contrast to the β-arrestin-2 knockout, tracheal rings from a GRK5 knockout mouse revealed no differences in β_2AR-mediated relaxation compared with wild-type (23). Yet, in cell-based assays, GRK5 readily phosphorylates the receptor in an agonist-dependent fashion and evokes receptor uncoupling to the cAMP response (24). These data suggest that GRK2, which is also expressed in airway smooth muscle at levels similar to GRK5, may be sufficient to carry out this function in the GRK5 knockout.

In conclusion, we have delineated the relative effects of PKA and GRK-mediated phosphorylation of β_2AR on receptor function in a physiologic system. In airways expressing mutated β_2ARs lacking kinase phosphorylation sites, acute β-agonist responsiveness was increased, indicating that both events act as rapid attenuators of the bronchodilatory response to β-agonists. Thus, they are an integral component of the initial response to receptor activation. The extent of receptor occupancy by agonist is a critical determinant of the physiologic impact of each kinase. Thus, these events need to be considered in the context of the bronchodilatory response to acute administration of β_2AR agonists in previously untreated patients and not simply in terms of long-term agonist-promoted desensitization/tachyphylaxis. Based on these results, it appears that modulation of these attenuator functions, with adjunct therapies, or biased ligands, may improve acute β-agonist treatment of obstructive lung disease.

Materials and Methods

Transgenic Mice. All experiments were performed in accordance with protocols approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Site-directed mutagenesis using methods previously described (24) was used to alter the human WT β_2AR cDNA. For the β_2PKA(-) construct, codons were mutated representing Ser-261, 262, 345, and 346 to result in Ala. For the β_2GRK(-) construct, codons representing Ser or Thr at amino acid positions 355, 356, 360, 364, 384, 393, 396, 401, 407, 408, and 411 were mutated so that there was a substitution of Ala at all sites. The β_2PKA(-)/GRK(-) construct had all of the above substitutions. To achieve cell type-specific expression in mice, the full-length β_2AR constructs were subcloned into a vector containing a 5′ smooth muscle α-actin promoter fragment (termed SMP-8) in addition to a 3′ SV40 polyadenylation signal as previously described (15). The entire 5.6-kb transgene was excised with *Not*I, purified, and injected into fertilized FVB/N eggs using standard procedures (15). The F0 and subsequent offspring were screened for incorporation of the transgene by PCR-based genotyping from tail-clip DNA with the following primers, designed to amplify the junction between β_2AR and the SV40 sequence: 5′-GCTTTCAATCCCTTATCTACTGCC-3′ and 5′-TAAAGGCATTCCACCACTGCTCCC-3′. F2-F8 offspring from transgenic mice expressing the β_2WT and the mutant with matched receptor protein expression levels were used for all studies.

Primary Airway Smooth Muscle Culture. Tracheas were excised, cut in a longitudinal fashion, and then cut in cross-sections of 2–3 mm. These were placed intima side down in sterile dishes and maintained in Dulbecco's modified Eagle's medium with 20% FCS and antibiotics. Explants were kept at 37 °C, 5% CO_2, 95% air for 3 days, and then serum-reduced to 10%. The outgrowing cells that adhered under these conditions were >95% airway smooth muscle, and after confluency were passaged at 1:4. Studies were performed with cells at 90% confluency, from passages 2–8. β_2AR expression was determined with solubilized cell membranes by western blots using an antibody that identifies both mouse and human receptors (Santa Cruz Biotechnology). RT-PCR was performed on total RNA with an oligo-dt RT primer and human-specific PCR primers: 5′-CCATCAACTGCTATGCCAATGAG-3′ and 5′-TATCCAGCCTGCTCCCCTGCGTTG-3′. Whole-cell cAMP was generated in 96-well plates (40,000 cells/well) in a manner analogous to the physiologic studies, with 5- or 30-min exposures to 10 nM or 30 μM ISO, respectively, and detected using a fluorescence-based assay (Molecular Devices). Results are normalized to the forskolin response.

Tracheal Ring Studies. Excised trachea were dissected free of surrounding tissue and cut to $\approx$5-mm cross-sections. These were studied with an ex vivo isometric myograph system (Radnoti) (15, 16). Briefly, rings were maintained in a bath of physiologic saline solution (118 mM NaCl, 4.73 mM KCl, 1.2 mM $MgCl_2$, 0.026 mM EDTA, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM NaH_2CO_3, and 11 mM glucose) at 37 °C and bubbled with 95% O_2, 5% CO_2 to maintain a pH of 7.4. The lumen of the trachea was stretched between the wires of the transducers to a tension of 5 mN. After equilibration and readjustment of baseline tension, trachea were exposed to the various agents, and the tension recorded from the analog to digital converter. Contraction was measured after multiple doses of acetylcholine, and the EC_{50} for acetylcholine contraction was determined. After washout and return to baseline contraction, rings were contracted by continuous exposure to the EC_{50} dose of acetylcholine for the remainder of the experiment, and ISO was added to the bath at the indicated concentrations. The extent of relaxation to ISO was stable within 5 min after addition of the agonist, and the force was recorded for a given dose at this 5-min time point. Dose-response curves were initially compared by

ANOVA, followed by posthoc *t*-tests of the responses at specific concentrations (see text).

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